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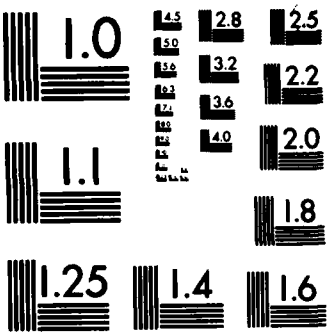

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THE DERIVATIZATION OF POLYSTYRENE AND NYLON BEADS. A CONTROLLED INTRODUCTION OF FUNCTIONAL GROUPS FOR IMMOBILIZATION OF ANTIBODY PROTEIN

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SUMMARY

An attempt was made to modify the surface properties of nylon balls and polystyrene tubes so that protein could be covalently attached. Using ^{125}I labeled antibody as our probe to quantitate the amount of protein bound to the solid support, the modified solid supports immobilized from [illegible] more antibody than the non-modified supports. Also, our data show that glutaraldehyde was [illegible] more effective than carbodiimide as a coupling reagent for antibody immobilization. It is observed that receptors for antibody protein on either the modified or unmodified supports remain [illegible] at every level of antibody protein concentration used to sensitize the supports, which in practice, permits nonspecific uptake of other protein molecules subsequently used in reaction identification. This causes difficulty in test interpretation. Work is in progress to determine if the unbound receptor sites can be effectively blocked without reducing the immunoreactivity of the immobilized antibody.

INTRODUCTION

Several methods of amplifying the antigen-antibody reaction have been introduced through the years. Of these methods, enzyme based immunoassays have gained widespread use. The majority of the enzyme-linked immunoassays are based on simple physical adsorption of antigen (or antibody) to plastic surfaces.[1] The disadvantages of simple adsorption have become numerous. The problems encountered are: nonspecific adsorption; desorption; incomplete surface coverage leaving uncoated areas as potential receptors for nonspecific binding of test reagents; limited capacity for antigen or antibody adsorption; lack of reproducibility; and variation in binding characteristics of plastic supports, even if the same lot number or different wells of the same microtiter trays are used.

The need for an improved carrier support for antigen detection of infectious diseases is well known to investigators active in enzyme immunoassays. Significant advances have been made in derivatization of various plastic tubes or balls by introduction of functional groups of defined biochemical reactivity.[2] If one could covalently link an antibody onto a carrier support without significantly reducing the antibody's specific activity, many of the above mentioned disadvantages of simple adsorption would be eliminated.

We have compared simple adsorption of antibody to plastics and nylon carrier supports with those that have been derivatized to covalently bind antibody protein. This report summarizes the results of these studies.

MATERIALS AND METHODS

Microtrays: 96 well flat bottom (Dynatech MicroElisa plate, Lot #2979, Falcon Lot #D1431960, Becton Dickinson Co. and Costar Lot #C20A1359, Rochester Scientific).

Plastic balls: 1/4" polystyrene balls (Precision Plastic Ball Co., Chicago, Ill).

Nylon balls: 1/4" nylon balls (Precision Plastic Ball Co., Chicago, Ill).

Wash buffer: PBS/Tween pH 7.4, (40 g NaCL, 1g KH_2PO_4, 14.5 g Na_2HPO_4.12 H2O, 1g KCL, 2.5 ml Tween 20, 1g NaN_3, made up to 5 liters with distilled water.)

Coating buffer: Carbonate/bicarbonate buffer pH 9.6 (1.59 g Na_2CO_3, 2.93 g $NaHCO_3$, and .2 g NaN_3, made up to one liter with distilled water. Prepare fresh each week).

Derivatization of Plastic: Derivatization of either the plates or plastic balls was accomplished by the procedure of Neurath and Strick[3]. Briefly, microplates or balls were placed on a well ventilated hood and filled (covered) with methanesulfonic acid. After overnight incubation at room temperature, the plates (balls) were extensively washed with distilled water and dried. The plates (balls) were then filled (covered) with a mixture of glacial acetic acid/fuming nitric acid (1:1) and placed in a 40°C incubator. After 4 hrs, the nitrated plates (balls) were extensively washed with tap water until the pH of the wash was 6.0 or higher. The plates (balls) were then filled (covered) with 0.5% sodium dithionite in 0.5M NaOH to convert polynitrostyrene to polyaminostyrene. After 1 hr, the plates (balls) were extensively washed with tap water. They were then treated with 1% glutaraldehyde in 0.05M Phosphate, pH 8.5 for 2 hrs at room temperature and 18 hrs at 6°C. The plates (balls) were then thoroughly washed with tap water and stored at 6°C with 0.1M PBS pH 7.4 until used.

Nylon balls: Derivatized nylon balls were prepared by hydrolyzing with 3N HCl as previously described[4] or with succinic anhydride[5].

Coupling of Goat anti-rabbit IgG to Nylon balls: Goat anti-rabbit IgG, IgG fraction (Miles-Yeda Lot #5-574) was radiolabeled with ^{125}I as described[6]. Nylon 6/6 balls (Aldrich Chemical) were hydrolyzed with HCl and activated with acetic anhydride and 1-cyclohexyl-3-(2-morpholinoethyl) carbodiimide Metho-p-toluene sulfonate (CMC) or glutaraldehyde, then coupled with antibody as described[3].

Coupling of Rabbit Anti Mycoplasma to Nylon beads: Rabbit anti mycoplasma serum (M.A. Products Lot #40986) was fractionated with 50% saturated ammonium sulfate. ^{125}I radiolabelling was carried out using the Chloramine-T method[6]. Activation of the nylon with succinic anhydride and CMC in 1,2, diaminoethane was done according to Kiefer[5]. Coupling to antibody (250 µg/ml per 6 balls) was effected by incubation of the derivatized nylon balls for 2 hrs at room temperature and 18 hrs at 6°C. The balls were then thoroughly washed with washing buffer.

Coupling of Antibody to derivatized microtiter plates or to polysytrene balls: The coupling procedure is essentially the same for either the derivatized microtiter plates or plastic balls. 100 µl of antibody dilution (100 µg/ml in coupling buffer) were pipetted into each well of a microtiter plate. The plate was allowed to incubate at room temperature for 1-2 hours, then at 6°C overnight. It was then rinsed several times with washing buffer and used (for our purpose) within 1 week. Plates and plastic balls were stored in washing buffer at 6°C until used.

Methods used to block remaining active groups: Either 1% bovine serum albumin or a .5M solution of glycine was added to each well of a microtiter tray (or to balls) to saturate any receptor sites on the carrier supports that were not taken up by specific antibody. After 2 hrs at room temperature, the trays (balls) were washed once with wash buffer and used immediately.

The ELISA test: 100 µl of each test antigen were added to a well (ball) and allowed to combine with antibody for 2 hrs at room temperature. Trays (balls) were then washed with washing buffer 3 times. A second antibody (animal species different from the one used to sensitize the carrier supports in this instance, goat) was then added to form antigen/antibody complexes with the captured antigen. The plates (balls) were allowed to incubate 1 hr at room temperature, then free antibody removed by washing 3 times with washing buffer. Alkaline-phosphatase conjugated rabbit anti goat antibody was diluted 1:500 in PBS/Tween and 100 µl were added to each well (ball). After 1 hr at room temperature, the plate (balls) were washed 3 times with washing buffer and .2 ml of a solution of substrate, p-nitrophenylphosphate (Sigma Co. St. Louis) in 10% diethanolamine buffer pH 9.8 were added. The enzyme reaction was allowed to develop for 15 minutes at room temperature. Readings at 405 nm were taken using a Dynatech MICRO ELISA Reader.

Results:

A quantitative study was done with nylon balls to determine the amount of antibody protein uptake and to compare the uptake between nylon that had been "activated" vs. "non-activated" nylon balls. In this study, activation was effected by acid hydrolysis and glutaraldehyde treatment. The data in Table I summarizes these studies. As the data show, the hydrolysed glutaraldehyde treated "activated" nylon took up from 2.3 - 5.2 times more antibody protein than "non-activated" nylon. In another study using succinic anhydride/CMC to activate the nylon balls for antibody protein attachment, it was again demonstrated that the "activated" nylon would take up at least twice the amount of antibody protein as the non-activated nylon (Table II).

To further determine the optimal method of nylon derivatization, nylon was treated with acetic anhydride using CMC as the bifunctional reagent, the data from this experiment is shown in Table III. The data show that less antibody protein was immobilized by derviatization with acetic anhydride than when treated by acid hydrolysis and glutaraldehyde.

Derivatization of the microtiter plate to a polyaminostyrene provided a carrier support that gave uniform and reproducible binding of antibody protein when compared to the untreated commercially available plates. Such treatment eliminated well to well and plate to plate variation in antibody protein uptake. The microtiter plates that were used without treatment, with rare exception, failed to provide reliable and reproducible results.

TABLE I

Comparison of antibody uptake between nylon balls that had been "activated" by hydrolysis to "nonactivated" nylon balls

Conc. of Goat Anti Rabbit IgG µg/ml	UPTAKE OF ANTIBODY ONTO NYLON (ng/mm^2)		RATIO OF ACTIVATED
	NONACTIVATED	ACTIVATED	NONACTIVATED
50	9.1	21	2.3
100	11	32	3.0
200	13	66	5.2
400	22	73	3.4

TABLE II

Comparison of antibody uptake between nylon balls activated with succinic anhydride and CMC to the amount of antibody taken up by nonactivated nylon balls

Conc. of Rabbit Mycoplasma (µg/ml) antibody	UPTAKE OF ANTIBODY ONTO NYLON (ng/mm^2)		RATIO OF TREATED
	UNTREATED	TREATED	UNTREATED
250	52	106	2.0

TABLE III

Antibody uptake of nylon balls "treated" with acetic anhydride and CMC compared to antibody uptake of "nontreated" nylon balls

Conc. of Goat Anti Rabbit IgG (μg/ml)	UPTAKE OF ANTIBODY ONTO NYLON (ng/mm^2)		RATIO OF TREATED
	UNTREATED	TREATED	UNTREATED
50	7.0	12	1.7
100	8.2	15	1.8
200	8.6	17	2.0
400	16	26	1.6

DISCUSSION:

Application of immunochemical methods for the identification of microbial antigens and antibodies has gained widespread interest during the past decade. Radioimmunoassays (RIA) are very sensitive and simple to perform but have the disadvantage of requiring sophisticated equipment and the use of potentially hazardous reagents. Recently, the utilization of enzyme to label antibodies for use in antibody protein assays has become generally accepted as an alternative for, and even a substitute, for the RIA and other immunological assays. The extension of the use of the enzyme immunoassays (called the ELISA test) to identify bacterial and viral infections has emphasized the need for a method for immobilizing antibody to a carrier support without loss of its high antigen binding capacity. Catt and Tregear[7] proposed the use of a solid support to bind an antibody for use in RIA, and refined this technique to use polystyrene tubes.

Polystyrene tubes and microtiter trays have been widely used in the ELISA test to determine antibody titer to a large number of bacterial/viral agents[8]. Polystyrene has not been as widely used to detect antigen due to the nonspecific nature of the binding of antibody to its surface resulting in high background readings and difficult interpretations.

Our objective was to introduce specific functional groups on to the carrier supports so that antibody would be linked covalently. Our initial studies indicate only partial success. It is apparent from our data that only partial surface coverage occurs whether one covalently links antibody to a solid support or whether physical adsorption of antibody occurs. The data suggest that derivatization allowed an uptake of over 5 times as much antibody as physical adsorption. However, functional groups still remained available for subsequent uptake of other protein molecules which could contribute to difficulty in test interpretation. Various agents are being tested for their use in blocking "nonspecific" uptake of protein from clinical samples under test.

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An attempt was made to "modify" the surface properties of nylon balls and polystyrene tubes so that antibody protein could be covalently attached. Using ^{125}I labeled antibody as our probe to quantitate the amount of protein "bound" to the solid support, the "modified" solid supports immobilized from 2-5 times more antibody than the non-modified supports. Also, our data show that glutaraldehyde was 2-4 times more effective than carbodiimide as a coupling reagent for antibody immobilization. It is apparent that receptors for antibody

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protein on either the modified or unmodified supports remain "free" or "unbound" at every level of antibody protein concentration used to sensitize the supports, which in practice, permits "nonspecific" uptake of other protein molecules subsequently used in reaction identification. This causes difficulty in test interpretation. Work is in progress to determine if the "unbound" receptor sites can be effectively blocked without reducing the immunoreactivity of the immobilized antibody.